Applications of Oblique-Incidence Reflectivity Difference Method in Primary Study of Protein Biomolecules *

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Oblique-incidence reflectivity difference (OI-RD) analysis is applied to detect the immunoglobulin-G and cytochrome biomolecules on standard glass substrates without fluorescence labelling. The OI-RD intensities not only depend on the protein structure, but also vary with the protein concentration. The results indicate that this method should have potential applications in detection of biochemical processes.

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Typically, the interactions of biomolecules are investigated by fluorescence spectroscopy in which fluorescent labelling is often necessary.[1] As the labelling procedure is complicated and could cause some damage or modifications in the structure and biological activity of biomolecules, it would be preferable to have some method to detect the interaction without labelling. Recently, label-free methods for protein microarrays include surface plasmon resonance microscopy,[2] mass spectrometry[3] and oblique-incidence reflectivity difference (OI-RD) analysis.[4] The sensitivity of surface plasmon resonance microscopy derives from the sharp resonance of the plasmon surface polariton, so that this method requires microarrays to be fabricated on functionalized gold films. Mass spectrometry requires that the biomolecules be fabricated upon a special matrix medium for laser-induced desorption and ionization. By comparison, the OI-RD method requires only optically flat substrates and can be used to measure a large number of samples rapidly.[5−7]

The OI-RD technique is a particular form of optical ellipsometry used to directly measure small changes in the optical properties of thin films,[8,9] and has been demonstrated to be sensitive to a relative change in reflectivity of $\Delta R/R = 1 \times 10^{-5}$ and a change in coverage of $\Delta\theta = 0.02$.[10,11] As an in situ probe method, it has been commonly applied to detect the interaction of biomolecules. At oblique incidence the reflectivities of p- and s-polarized light change disproportionately in response to the thickness and/or complex dielectric constant of a film change. Let r_{p0} and r_{s0} be the reflectivities on a bare surface for p- and s-polarized light, respectively, r_p and r_s the reflectivities on the surface covered with a thin film, and $\Delta p = (r_p - r_{p0})$ and $\Delta s = (r_s - r_{s0})$. The OI-RD technique enables direct measurements of the real $[\mathrm{Re}(\Delta p - \Delta s)]$ and imaginary $[\mathrm{Im}(\Delta p - \Delta s)]$ to be carried out. In terms of the ellipsometric ratio $\rho = r_p - r_s = \tan\psi \exp(i\delta)$, it is easily seen that $\Delta p - \Delta s \approx (\rho - \rho_0)/\rho_0$, so that $\mathrm{Im}(\Delta p - \Delta s) \approx \delta - \delta_0$ and $\mathrm{Re}(\Delta p - \Delta s) \approx 2\csc[\psi_0(\psi - \psi_0)]$. The equation for $\mathrm{Im}(\Delta p - \Delta s)$ given in Refs. [4–7] indicates that it depends on the incidence angle θ and the optical dielectric constants ε_0, ε_d and ε_s of the ambient medium, film, and substrate, respectively. For a He-Ne laser wavelength of 632 nm, $\varepsilon_0 = 1$ (air), $\varepsilon_s = 2.31$ (glass), and so $\mathrm{Im}(\Delta p - \Delta s) \neq 0$.

The layout of our OI-RD system is shown in Fig. 1. A p-polarized He-Ne laser beam passes through a polarization modulator (modulation frequency $\Omega = 50$ kHz). The modulator causes the output beam to oscillate between p- and s-polarizations with elliptically polarized intermediate states. The polarization-modulated beam then passes through a Pockels cell that introduces an adjustable phase ϕ_0 between the s- and p-polarized components. The resultant beam is focused to a 10 μm spot on a protein surface at an incidence angle $\theta = 72°$. After reflection and recollimation, the beam passes through a rotatable analyser. The intensity of the transmitted beam, $I_R(t)$, which consists of various harmonics of the modulation frequency Ω is detected with a photodiode and Fourier analysed with digital lock-in amplifiers. We detect the first and second harmonic amplitudes, $I(\Omega)$ and $I(2\Omega)$. Initially the sample is mounted in a po-

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sition where the incident beam reflects off the bare surface of the glass substrate. We adjust the analyser to zero $I(2\Omega)$ and then adjust the phase (with the Pockels cell) to zero $I(\Omega)$. The proportionality constants are measured separately so that $\Delta p - \Delta s$ is determined absolutely. We monitor the behaviour of the intensity of $\mathrm{Im}(\Delta p - \Delta s)$, which changes according to the biomolecular characteristics. The sample is prepared as spots about 18 mm × 18 mm in size on glass plates. The laser is incident at the centre of the spot in all measurements.

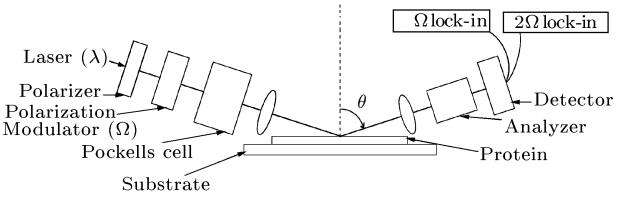

Fig. 1. Optical layout of the OI-RD system with protein thickness d.

To evaluate the experimental error of the measurement, the $\mathrm{Im}(\Delta p - \Delta s)$ intensities of three random spots without any protein on one glass plate were measured over a given period of time, and the results are shown in Fig. 2. The figure indicates that the maximal noise of the three spots is 2.0×10^{-4} and the maximal error among them is 4.0×10^{-4}.

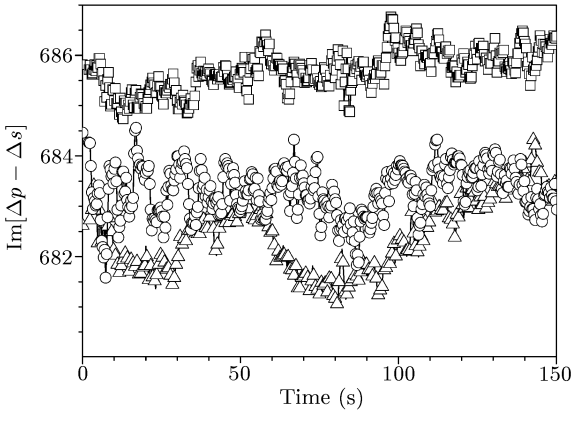

Fig. 2. $\mathrm{Im}(\Delta p - \Delta s)$ intensities of three spots on the bare glass. Open squares: first spot; open triangles: second spot; open circles: third spot.

The OI-RD intensities for two biomolecules, immunoglobulin-G (IgG) and cytochrome (Cytc), of concentrations 500, 50 and 5 μg/ml were measured. The intensities of $\mathrm{Im}(\Delta p - \Delta s)$ of the two proteins versus concentration are shown in Fig. 3. In this figure the $\mathrm{Im}(\Delta p - \Delta s)$ intensities of the samples vary from 1.1×10^{-1} to 2.2×10^{-1}. Compared Fig. 2 with Fig. 3, it can be found that the $\mathrm{Im}(\Delta p - \Delta s)$ intensity of the protein is much larger than that of the background noise, thus OI-RD can be used to monitor the protein on the glass substrate.

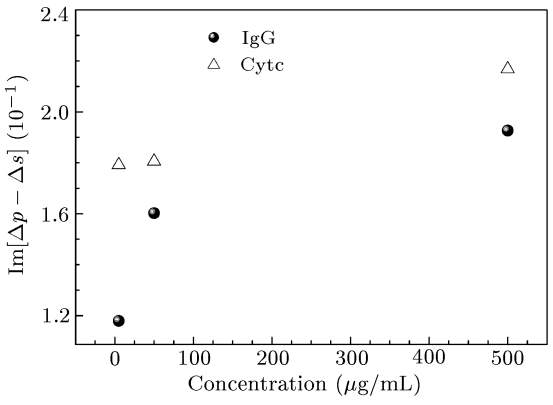

Fig. 3. $\mathrm{Im}(\Delta p - \Delta s)$ intensity versus concentration for the proteins IgG and Cytc. Circles: IgG; triangles: Cytc.

From the figures we can see that the intensity of $\mathrm{Im}(\Delta p - \Delta s)$ for Cytc is larger than that of IgG, for all concentrations measured. For both proteins, the intensity varies with concentration and increases with larger concentration, but the rates of change are slightly different. This might be due to the different characteristics of different biomolecules. The shape of the intensity curve versus concentration for IgG is identical to that shown in Ref. [6].

In conclusion, we have demonstrated that the OI-RD technique can be used to detect biomolecule spots without any labelling agent. The type and concentration of the biomolecule can be specified qualitatively by its $\mathrm{Im}(\Delta p - \Delta s)$ intensity. The present study proves that the OI-RD method can be affectively applied to detect biochemical processes.

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